

# **Document made available under the Patent Cooperation Treaty (PCT)**

International application number: PCT/IL05/000105

International filing date: 27 January 2005 (27.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US  
Number: 60/539,570  
Filing date: 29 January 2004 (29.01.2004)

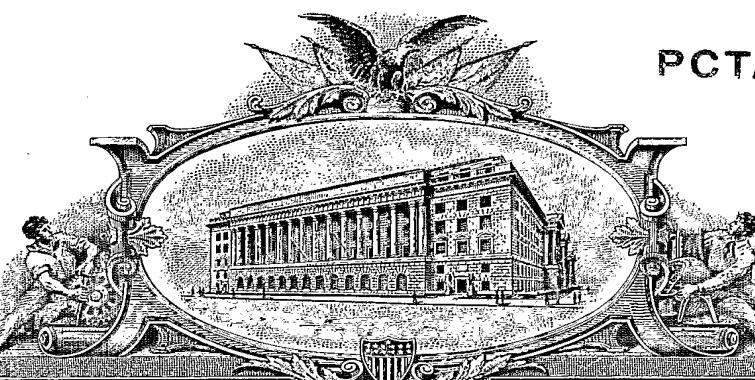
Date of receipt at the International Bureau: 21 March 2005 (21.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland  
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PA 1280201



THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:  
UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 16, 2005

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM  
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK  
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT  
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A  
FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/539,570  
FILING DATE: January 29, 2004

By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS



M. K. HAWKINS  
Certifying Officer

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

**PROVISIONAL APPLICATION FOR PATENT COVER SHEET**

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. \_\_\_\_\_

13441  
60/536570

012904

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Jacob	HOCHMAN	Jerusalem, ISRAEL
Additional inventors are being named on the _____ separately numbered sheets attached hereto		
TITLE OF THE INVENTION (500 characters max)		
DIAGNOSIS, PROGNOSIS AND TREATMENT OF CANCER		
Direct all correspondence to: CORRESPONDENCE ADDRESS		
<input checked="" type="checkbox"/> Customer Number:	20529	
OR		
<input type="checkbox"/> Firm or Individual Name		
Address		
Address		
City	State	Zip
Country	Telephone	Fax
ENCLOSED APPLICATION PARTS (check all that apply)		
<input checked="" type="checkbox"/> Specification Number of Pages	15	
<input checked="" type="checkbox"/> Drawing(s) Number of Sheets	3 in color	
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76		
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT		
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.	FILING FEE Amount (\$)	
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees.		
<input checked="" type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 14-0112	\$ 80.00	
<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.		
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.		
<input checked="" type="checkbox"/> No.		
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: _____		

[Page 1 of 2]

Respectfully submitted,

SIGNATURE 

TYPED or PRINTED NAME Lee C. Heiman

TELEPHONE 202-775-8383

Date January 29, 2004

REGISTRATION NO. 41,827

(if appropriate)

Docket Number: 25963

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

2002-09  
13441 U.S. PTO  
10761

MAIL STOP PROVISIONAL PATENT APPLICATION  
Attorney Docket No. 25963

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

13441 U.S. PTO  
60/539570

012904

In re Application of:

Jacob HOCHMAN

Serial No. NOT YET ASSIGNED

Filed: January 29, 2004

For: **DIAGNOSIS, PROGNOSIS AND TREATMENT OF CANCER**

**TRANSMITTAL LETTER**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

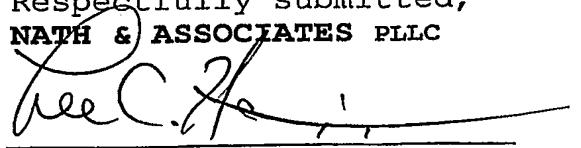
Submitted herewith for filing in the U.S. Patent and Trademark Office is the following **PROVISIONAL APPLICATION**:

- (1) Transmittal Letter
- (2) Cover sheet for filing Provisional Application
- (3) 18 page Provisional Application consisting of:
  - 15 pages Textual Specification,
  - 0 pages of Claims,
  - 0 page of the Abstract,
  - 3 sheets of Drawings in color;
- (4) Check No. 20280 \$ 80.00 for filing fee as a small entity;
- (5) Postcard for early notification of serial number.

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,  
**NATH & ASSOCIATES PLLC**

By:

  
Gary M. Nath  
Registration No. 26,965  
Lee C. Heiman  
Registration No. 41,827  
Customer No. 20529

Date: January 29, 2004  
NATH & ASSOCIATES PLLC  
1030<sup>th</sup> 15<sup>th</sup> Street, NW - 6<sup>th</sup> Floor  
Washington, D.C. 20005  
GMN/LCH/dd:APPL.trans

## DIAGNOSIS, PROGNOSIS AND TREATMENT OF CANCER

### FIELD OF THE INVENTION

This invention relates to methods for diagnosis, prognosis and treatment of cancer.

### BACKGROUND OF THE INVENTION

Mouse Mammary Tumor Virus (MMTV) is a B-type slowly transforming retrovirus, associated primarily with the induction of mammary carcinomas in laboratory mice. The most documented association of MMTV with non-mammary tumors is that with T-cell lymphomas [1,2].

Two nuclear proteins have been described in T-cell lymphomas that harbor MMTV [3]. These proteins are named p14 and p21 and were identified by a monoclonal antibody (M-66) generated against a cell-surface epitope of an immunogenic cell variant of the S49 T-cell lymphoma (named **T-25-Adh**). T-25 Adh cells are immunogenic, non-tumorigenic cells that grow as an adherent monolayer. They were derived from a highly tumorigenic variant of the S49 lymphoma, named **T-25**. T-25 cells grow in suspension culture as single cells. Priming of immune competent, syngeneic Balb/C mice with T-25-Adh cells protects them for a lifetime against a subsequent challenge with T-25 cells [4]. The median survival of naive mice inoculated with T-25 cells alone is 14-16 days. T-25-Adh cells express solely the p14 whereas T-25 cells express both p14 and p21 [3].

The leader peptide (p14) of the MMTV Env precursor has recently been purified sequenced and identified [5]. It binds (based on co-localization and co-immunoprecipitation studies) to the shuttling protein B23, implicated in both transport and growth regulation, as well as in other cellular functions [6, 7]. Association with B23 has been previously reported for other auxiliary nucleolar

retroviral proteins, such as Rev (HIV) and Rex (HTLV). This leader protein, p14, interacts with the monoclonal antibody M-66 and the epitope recognized by this antibody has been identified [5].

Figure 1 summarizes the sequence and putative modification sites of p14, and relationship to the MMTV-Env precursor.

Additional interest in MMTV has surfaced in the last years when new evidence suggested an MMTV-like retroviral association with human breast cancer [9]. In these studies, MMTV-like Env gene sequences that were 95-99% homologous to mice MMTV sequences, were found in 121 (38.5%) of 314 human breast tumor samples analyzed. Additional support for the involvement of MMTV in human breast cancer came from another group attempting to detect such sequences in human breast tumors. These investigators [10] have detected the presence of MMTV-like Env sequences in 37% of the breast tumors analyzed. DNA sequencing has shown these sequences to be 99-100% homologous to MMTV Env gene sequences present in inbred mice. These sequences have not been detected in normal breast tissue [10]. Furthermore, these sequences have been detected in a T-cell lymphoma of a breast cancer patient who was simultaneously diagnosed with both diseases [10]. A third group [11] has recently reported the presence of MMTV-like gene sequences in 42.2% (19 of 45) archival breast cancer biopsy tissues from Caucasian-Australian women, but only 1 of 120 (0.8%) from Vietnamese women. Liu *et al.* [12] have recently reported finding a complete proviral MMTV structure in the genome of human breast cancer tissues. There exists an 85% identity of the translated 5'-terminal env sequence (Genbank accession number AF248270) reported by this group with the p14 amino acid sequence (Fig. 2).

## DESCRIPTION OF THE INVENTION

The present invention is based on the following unexpected results:

- (a) Polyclonal antibodies specific to p14 (PAb-14) were generated in rabbits (by the use of a recombinant p14) and it has been found that PAb-14

recognize p14 in frozen as well as in paraffin embedded sections of different S49 derived tumors. This finding came as a surprise particularly in view of the fact that the monoclonal antibody M-66 does not recognize tumors in frozen or paraffin embedded sections.

(b) Using the PAb-14 it has now been established that mouse mammary carcinoma cells harboring MMTV also demonstrate p14 in the nucleoli.

(c) It has also been found that there is a strong correlation between the level of expression of p14 in S49 cells and the severity of the disease. Thus, T-25 cells express a higher level of p14 than T-25-Adh cells.

(d) Using commercially available paraffin embedded sections of human breast cancer samples subjected to immunohistochemical analysis using the PAb-14 and peroxidase-labeled second antibodies it has been found that of 25 different breast cancer samples tested (all on the same slide) one gave an extremely strong signal and three gave lighter but distinctive signals.

The combination of the above results paves the way to the establishment of methods for early diagnosis and prognosis of human cancer and in preference, of human breast cancer. Further, the present invention paves the way to the establishment of methods for treatment of cancer.

Thus, the present invention concerns the use of anti-p14 or anti-p21 *binding agents* for diagnosis as well as prognosis of human cancer. According to one embodiment, the diagnosis/prognosis may be achieved by the use of an appropriate kit, such as those based on immunofluorescence, immunoperoxidase, ELISA type assays, Western blotting etc. According to the above unexpected finding, the kit may be applied on both fresh biopsies as well as on frozen and paraffin embedded sections and tissues.

The present invention also provides a method for the treatment of cancer. According to one embodiment, the method concerns the administration

to a patient in need a therapeutically effective amount of one or more anti-p14 and/or anti-p21 binding agent.

The anti-p14 or anti-p21 binding agents are any agent which specifically binds to the protein or to a functional fragment thereof and affects its expression in the cell (and thereby its viability). The binding agent may be an antibody which specifically binds to the protein, or it may be a specific siRNA, a specific low molecular weight compound or a specific peptide.

The antibodies may be (a) antibodies which specifically bind the protein; (b) fragments of the antibodies of (a) which substantially retain the antigen binding characteristics of the whole antibody; and (c) antibodies which specifically bind to an antigenic epitope bound by any one of the antibodies of (a) and (b) above. The antibodies may be of any of the classes IgG, IgM, IgD, IgA and IgE.

The antibodies can be polyclonal or monoclonal.

Although, typically, the anti-p14 or anti-p21 antibodies are produced by hybridoma cell lines, they may also be produced by recombinant genetic methods. The antibody may be animal derived, typically a mouse antibody as well as a human antibody, a chimeric antibody, a "*humanized antibody*", a primatized antibody, etc.

Fragments and chimeras of the antibodies which fall within the scope of the present invention are such which substantially retain the Ag binding characteristics of the whole antibodies from which they are derived and thus specifically bind to the protein.

The term "*substantially retain*" should be understood to mean that the binding affinity of the antibody fragment for the protein p14 or p21 as determined by any of the methods mentioned below is at least 50% of the binding affinity of the whole antibody for the same protein.

Antibodies may also be produced by inducing *in vivo* production in the appropriate lymphocyte population or by screening recombinant

immunoglobulin libraries in accordance with known methods which are described, for example, in Orlandi *et al.* *Proc. Natl Acad. Sci. USA* **86**:3833, (1989).

Antibodies may also be produced by DNA immunization with plasmids containing the cDNA of p14 or of p21. Such DNA immunizing methods are described, for example, in *Annu. Rev. Immunol.*, **15**:617, (1997).

According to a preferred embodiment of the invention the anti-p14 and/or anti-p21 binding agents are used for the treatment of carcinomas. While hitherto p14 was described in association with the manifestation of lymphoma, it has only now been established that p14 is also expressed and detected in a mammary carcinoma. This finding led to the establishment of the method of the invention of treatment of cancer, preferably, of carcinoma origin. This finding was unexpected in view of previous results showing that mammary carcinoma does not express p14 [Hoch-Marchain et al *Virology* **242**:246-254 (1998)].

According to a further preferred embodiment, the anti-p14 and/or anti-p21 binding agents are used in the treatment of breast cancer, with preference to treatment of sub-populations of breast cancer cases exhibiting expression of the p14 protein.

The treatment according to the invention can be carried out by any conventional treatment protocol, for example, through direct introduction of the binding agents or through binding agents coupled to toxins.

For example, cancer treatments based on p14 and/or p21 include: A-use of anti-p14 binding agents (e.g. humanized anti-p14 or anti-p21 antibodies) similar to the use of: a-the humanized anti-HER-2/neu herceptin that has been approved for use in patients with metastatic breast cancer that demonstrate overexpression of HER-2/neu; and b-the monoclonal antibody Rituxan that binds to the CD20 antigen present on B cell lymphomas and is currently approved for treatment of patients with relapsed or refractory low-grade CD20 positive follicular lymphoma [For review see Kim J.A. "Targeted therapies for the treatment of cancer; *American J. of Surgery* **186** (2003) 264-268]. Such

humanized anti-p14 or anti-p21 binding agents may be used either alone or in combination with cytotoxic chemotherapy. **B**-use of anti-p14 or anti-p21 binding agents (e.g. humanized anti-p14 antibodies) coupled to toxins or radioactive isotopes. **C**- generation of p14 or p21 specific cytotoxic T-cells, their expansion and use in adoptive transfer of immunization. **D**-use of combinatorial peptide libraries in order to select and isolate peptides that recognize p14 and/or p21 with high affinity, and subsequently use these peptides in targeted therapy (when coupled to toxins or radioactive isotopes).

#### **BRIEF DESCRIPTION OF THE FIGURES**

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

**Fig. 1** presents the sequence and putative modification sites of p14, and relationship to the MMTV-Env precursor.

**Figure 2** presents a comparison of the amino acid sequence of p14 with the sequence of the human proviral structure reported by Liu et. al. (12).

**Figure 3** presents the nucleolar localization of p14 in mouse mammary carcinoma cells (**Mm5MT**) in culture as shown by confocal microscopy.

#### **DESCRIPTION OF THE EXPERIMENTAL RESULTS**

##### **Polyclonal antibodies against p14**

Purified recombinant p14 has been prepared and used to immunize rabbits, and generate polyclonal antibodies against p14 (PAb-14).

Using the PAb-14, it was found by western blotting that p14 (but not p21- according to western analysis) is present also in the nucleoli of a mouse mammary carcinoma cell line (**Mm5MT**), received from the ATCC (Figure 3). This finding is significant as it implies that p14 may play a more general role

(unappreciated and unknown so far) not only in lymphomagenesis but also in mammary carcinogenesis induced by MMTV.

In addition to recognizing p14 (and p21) in western and immunofluorescence analysis PAb-14 recognized p14 (and p21) very specifically, in frozen tissue sections, as well as in paraffin embedded sections of different S49 derived tumors. Even S49 cell variants (named Rev-2-T-6) that metastasize to the brain [13, 14] were clearly identified in this tissue, which often shows a very high, non-specific background.

Since normal mouse brain tissue does not react with PAb-14 it has been established that anti-p14 antibodies (polyclonal as well as monoclonal) are very strong candidates for *in situ* identification of lymphomas and mammary carcinomas that harbor MMTV.

#### **Selective inhibition of p14 and p21 by siRNA**

RNA interference is an increasingly important method for functional genetic analysis and target validation. Tuschl and co-workers demonstrated that small interfering RNA duplexes (siRNA) can be transfected into mammalian cell lines, silencing gene expression and bypassing the non-specific interferon response [15, 16]. The importance of p14 to the phenotype of a series of T-cell lymphoma cells was tested by specifically silencing the synthesis of the protein at the level of the mRNA. Since the p14 and p21 proteins are products of integrated proviruses and there are many copies of the provirus integrated into the nucleus, it is not possible to know which copy is being transcribed and to silence it at the genomic level. Additionally, it cannot be excluded that p14 and p21 are transcribed from different proviral loci.

The expression of p14 in T-25 cells was successfully lowered for 24 hrs after transfection by electroporation with the siRNA. The levels of protein (according to western blot analysis) return to close to normal within 48 hrs.

## **Change of phenotype following transient up-regulation of p14 or p21 expression**

As a complimentary approach to gene silencing, the effect of over expression of p14 on the *in vitro* and *in vivo* phenotype of the specified cell lines was investigated. To this end the nucleotide sequence from the cDNA clone 66B (a cDNA clone containing the p14 sequence, isolated from a cDNA expression library of T-25-Adh cells by monoclonal antibody M-66 [3]) was used as a basis for cloning p14 into a mammalian expression plasmid (in the gateway system), as both a native protein and a C-terminal GFP fusion protein.

The C-terminal GFP fusion protein was transfected into T-25-Adh, T-25 and Rev-2-T-6 cells by electroporation. All cell lines were found to express GFP by FACS analysis within 24 hours of transfection. Some (~60% of viable cells) of the T-25-Adh cells were found to be growing in suspension after 24 hours. These cells were analyzed separately and found to have a higher level of fluorescence than cells remaining adherent. Thus at least a transient change in the adhesion phenotype of T-25-Adh cells was achievable.

### **Induced expression of p14/p21**

As a complimentary approach to gene silencing, the effect of over expression of p14 and p21 on the *in vitro* and *in vivo* phenotype of the cell lines was investigated. Stable cell lines over expressing p14 (or GFP-p14) seem to be difficult to establish (unpublished results). This may be due to a toxic effect of such large amounts of p14 or GFP or both. Thus, a tet-inducible plasmid was developed so that such effects would not be seen while establishing the cell line. Additionally, the plasmid express, also inducibly, GFP under the control of an IRES (commercially available from Clontech). On the one hand, expression of the GFP allows validating the presence of the plasmid and to localize the cells *in vivo*. On the other hand, as it is translated independently of the p14 and at lower levels, it does not affect p14 localization and function in the cell, nor it affects the cell by itself. This system allows to evaluate the effects of increasing p14 levels first *in vitro* and, subsequently, *in vivo* (as

described below) in a T-cell lymphoma as well as in other cell lines that do not harbor MMTV and, therefore, do not express p14 (such as the PIR-2 lymphoma and NIH 3T3 cells).

In vitro effects studies include intracellular localization of p14/21, doubling time, cell cycle distribution, adhesiveness, redistribution of p32, as well as other substrates to be identified. The use of naive transformed and non-transformed cell lines may be crucial in establishing whether p14 is in itself sufficient to increase and/or establish an oncogenic phenotype.

In vivo studies include (i) inoculation of 3 doses of over expressing T-25 cells vs. 3 doses of non-induced T-25 cells, and comparing their tumorigenicity [6(mice/group) x 3(doses) x 2(induced vs. non-induced cells) x 2(repetitions)=72 mice]; (ii) inoculation of 3 doses of over expressing T-25-Adh cells vs. 3 doses of non-induced control T-25-Adh cells, and comparing their ability to immunize these cells against a subsequent challenge with regular T-25 cells (72 mice, as in (i)); (iii) inoculation of 3 doses of over expressing Rev-2-T-6 cells vs. 3 doses of non-induced control Rev-2-T-6 cells, comparing their ability to metastasize to the brain and eyes (120 mice - 10 mice are needed per group in this experiment, as up to 70% of them demonstrate this metastasis under normal conditions).

Parallel experiments are performed following over expression of p21. This allows studying the effect of p21 on T-25-Adh or Rev-2-T-6 cells that normally do not express it, as well as on T-25 cells that express this protein endogenously.

In view of the fact that GFP, even at low levels, may influence the phenotype of cells, especially the complex interactions of the *in vivo* experiments proposed here, similar tet-inducible plasmids which do not express GFP and can be visualized *in vivo* using PAb-14 are developed.

Experiments using site-directed mutagenesis in an attempt to dissect the functionality of the protein sequence are also performed. The sequences investigated include the nuclear localization sequence (NLS), as well as putative modification sites (see fig. 1). Additionally, epitopes of interaction

between p14 (and p21) and co-isolating proteins, such as B23 or p32, are identified, and these epitopes are investigated as candidates for site-directed mutagenesis.

#### **Isolation and characterization of putative cellular substrates for p14**

In view of the findings that p14/p21 interact with B23, it was of interest to identify other putative substrates for this protein. To that effect, the nucleotide sequence from cDNA clone 66B was used as a basis for cloning p14 into a bacterial expression plasmid as an N-terminal His-tagged fusion protein. The purified recombinant His-tagged p14 was bound to an affinity column and subsequently used for affinity purification of whole cell extracts ( $3 \times 10^9$  cells/experiment). The proteins that remained bound to the p14 column after extensive washings were eluted, subjected to gel electrophoresis and microsequencing.

Using this approach B23 was again identified, thus strengthening the validity of this methodology. Another protein found to interact with p14 (in three independent experiments) using the affinity purification/electrophoresis/sequencing approach was p32 (gC1q-R). p32 is suggested to play a role in cell adhesion and tumor invasion and may be involved in nuclear - mitochondrial interactions. It is also likely to have a role in cellular signal transduction, as it is a substrate of mitogen activated protein (MAP) kinase. p32 is localized in mitochondria, cell surface and cytosol, but is translocated to the nucleus upon mitogenic stimulation [17-19]. Using a monoclonal antibody to p32, it was established that this protein is localized to the cytoplasm of T-25-Adh cells. However, in the tumorigenic T-25 cells p32 was seen mostly in the nucleus, and concentrated in the nucleolus.

Polyclonal antibodies against synthetic peptides that span the length of the proposed extension of p21 into gp52 (see Figure 1) were also generated. These antibodies seem to specifically recognize p21, albeit with low affinity.

### **Anti p14 antibodies in diagnosis and functional tumor inhibition studies**

For the purpose of conferring passive immunization against T-25 lymphoma cells that give systemic tumors, and/or against Rev-2-T-6 lymphoma cells that metastasize to the brain and eyes or against other lymphomas (EL-4) that harbor MMTV, antibodies are introduced every other day for 10 days following inoculation of the different cell lines into syngeneic mice that are followed thereafter for clinical signs as well as histopathological manifestations of tumor growth and metastasis. No. of mice used: 10(per group) x 2 (control and experimental) x 3(different cell lines) x 2(repetitions)=**120** mice.

For the purpose of conferring active immunization against T-25 and Rev-2-T-6 cells using purified p14 or p21, mice are primed and boosted with p14 and subsequently challenged with T-25 cells at different inoculi. They are followed for clinical and histopathological signs of tumor development. No. of mice used: 10(per group) x 3(different inoculi) x 2(repetitions) +10(control mice) x 2(repetitions)=**80** mice.

For the purpose of diagnosis murine mammary carcinomas that bear the MMTV virus, or human breast cancer cases that carry these DNA sequences, commercially available tissue (paraffin embedded) arrays from human breast cancer were acquired (Chemicon International, Select Tissue Array TMA1201) and were analyzed immunohistochemically. In particular, the commercially available paraffin embedded sections were subjected to immunohistochemical analysis using the polyclonal anti-p14 antibody and peroxidase labeled second antibodies (according to a standard protocol attached by chemicon to the tissue array samples). Of 25 different breast cancer samples tested (all on the same slide) one gave an extremely strong signal and three gave lighter, but distinctive signals. The rest were negative, as were five samples of normal human breast tissue analyzed on the same slide.

## **Discussion**

A multi-disciplinary approach is applied to investigate the role of the leader peptide of MMTV-Env precursor in lymphoma and mammary carcinoma, as this peptide is unexpectedly being translocated into the nucleoli in both malignancies. Understanding the molecular mechanisms involved, sheds new light on lymphomagenesis and mammary carcinogenesis induced by MMTV as well as offers potential new avenues for development of anti-lymphoma and anti mammary carcinoma treatments.

## References

1. Yanagawa, S., Kakimi, K., Tanaka, H., Murakami, A., Nakagawa, Y., Kubo, Y., Yamada, Y., Hiai, H., Kuribayashi, K., Masuda, T., and A., I. "mouse mammary tumor virus with rearranged long terminal repeats causes murine lymphomas." *J. Virol.*, 1993. **67**: p. 112-118.
2. Racevskis, J. "Altered mouse mammary tumor virus transcript synthesis in T-cell lymphoma cells." *J. Virol.*, 1990. **64**: p. 4043-4050.
3. Hoch-Marchaim, H., Hasson, T., Rorman, E., Cohen, S., and Hochman, J. "Nucleolar localization of mouse mammary tumor virus proteins in T-cell lymphomas." *Virology*, 1998. **242**(2): p. 246-54.
4. Hochman, J., Levy, E., Mador, N., Gottesman, M.M., Shearer, G.M., and Okon, E. "Cell adhesiveness is related to tumorigenicity in malignant lymphoid cells." *J Cell Biol*, 1984. **99**(4 Pt 1): p. 1282-8.
5. Hoch-Marchaim, H., Weiss, A.M., Bar-Sinai, A., Fromer, M., Adermann, K., and Hochman, J. "The leader peptide of MMTV Env precursor localizes to the nucleoli in MMTV-derived T cell lymphomas and interacts with nucleolar protein B23." *Virology*, 2003. **313**(1): p. 22-32.
6. Chan, P.K. "Characterization and cellular localization of nucleophosmin/B23 in HeLa cells treated with selected cytotoxic agents (studies of B23-translocation mechanism)." *Exp Cell Res*, 1992. **203**(1): p. 174-81.
7. Kondo, T., Minamino, N., Nagamura-Inoue, T., Matsumoto, M., Taniguchi, T., and Tanaka, N. "Identification and characterization of nucleophosmin/B23/numatrin which binds the anti-oncogenic transcription factor IRF-1 and manifests oncogenic activity." *Oncogene*, 1997. **15**(11): p. 1275-81.
8. Hiscox, J.A. "The nucleolus--a gateway to viral infection?" *Arch Virol*, 2002. **147**(6): p. 1077-89.
9. Wang, Y., Holland, J.F., Bleiweiss, I.J., Melana, S., Liu, X., Pelisson, I., Cantarella, A., Stellrecht, K., Mani, S., and Pogo, B.G. "Detection

of mammary tumor virus env gene-like sequences in human breast cancer." *Cancer Res*, 1995. **55**(22): p. 5173-9.

10. Etkind, P., Du, J., Khan, A., Pillitteri, J., and Wiernik, P.H. "Mouse mammary tumor virus-like ENV gene sequences in human breast tumors and in a lymphoma of a breast cancer patient." *Clin Cancer Res*, 2000. **6**(4): p. 1273-8.

11. Ford, C.E., Tran, D., Deng, Y., Ta, V.T., Rawlinson, W.D., and Lawson, J.S. "Mouse mammary tumor virus-like gene sequences in breast tumors of Australian and Vietnamese women." *Clin Cancer Res*, 2003. **9**(3): p. 1118-20.

12. Liu, B., Wang, Y., Melana, S.M., Pelisson, I., Najfeld, V., Holland, J.F., and Pogo, B.G. "Identification of a proviral structure in human breast cancer." *Cancer Res*, 2001. **61**(4): p. 1754-9.

13. Hochman, J., Assaf, N., Deckert-Schluter, M., Wiestler, O.D., and Pe'er, J. "Entry routes of malignant lymphoma into the brain and eyes in a mouse model." *Cancer Res*, 2001. **61**(13): p. 5242-7.

14. Assaf, N., Hasson, T., Hoch-Marchaim, H., Pe'er, J., Gnessin, H., Deckert-Schluter, M., Wiestler, O.D., and Hochman, J. "An experimental model for infiltration of malignant lymphoma to the eye and brain." *Virchows Arch*, 1997. **431**(6): p. 459-67.

15. Caplen, N.J., Parrish, S., Imani, F., Fire, A., and Morgan, R.A. "Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems." *Proc Natl Acad Sci U S A*, 2001. **98**(17): p. 9742-7.

16. Elbashir, S.M., Lendeckel, W., and Tuschl, T. "RNA interference is mediated by 21- and 22-nucleotide RNAs." *Genes Dev*, 2001. **15**(2): p. 188-200.

17. Majumdar, M., Meenakshi, J., Goswami, S.K., and Datta, K.U.-h.w.s.c.s.a.B.-V.-M.e. "Hyaluronan Binding Protein 1 (HABP1)/C1QBP/p32 Is an Endogenous Substrate for MAP Kinase and Is Translocated to the

Nucleus upon Mitogenic Stimulation." *Biochemical and Biophysical Research Communications*, 2002. 291(4): p. 829-837.

18. Day, A.J. and Prestwich, G.D. "Hyaluronan-binding proteins: tying up the giant." *J Biol Chem*, 2002. 277(7): p. 4585-8.

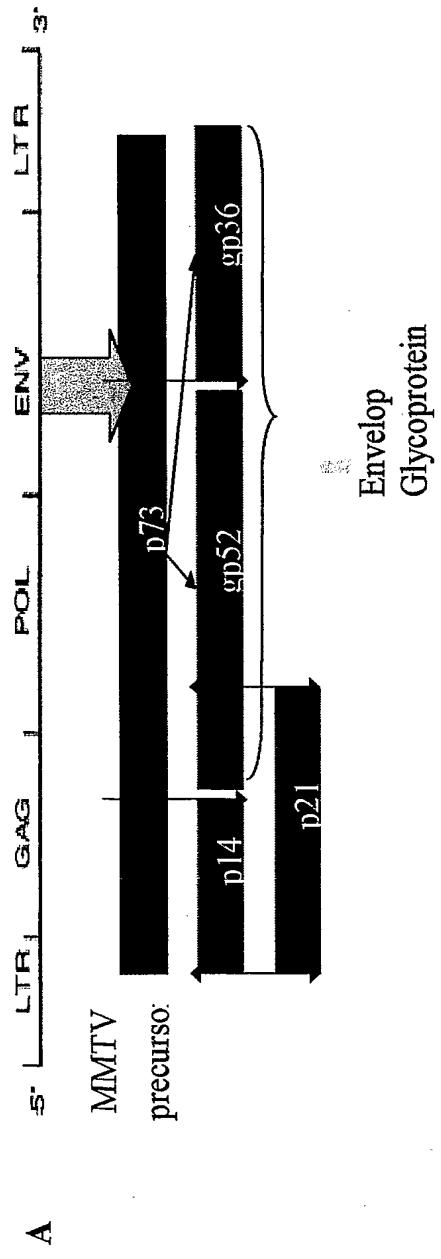
19. Meenakshi, J., Anupama, Goswami, S.K., and Datta, K. "Constitutive expression of hyaluronan binding protein 1 (HABP1/p32/gC1qR) in normal fibroblast cells perturbs its growth characteristics and induces apoptosis." *Biochem Biophys Res Commun*, 2003. 300(3): p. 686-93.

20. van de Wetering, M., Oving, I., Muncan, V., Pon Fong, M.T., Brantjes, H., van Leenen, D., Holstege, F.C., Brummelkamp, T.R., Agami, R., and Clevers, H. "Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector." *EMBO Rep*, 2003. 4(6): p. 609-15.

21. Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. "Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate." *Embo J*, 2001. 20(23): p. 6877-88.



0796EG/09  
O'DSUN 143



**B**

1 MPNHQS **GSP**T GSSD **LILSGK** KQR **PHILARR** KRREMKKIN RKVRRMNL DI

51 I**KERTAMONL** Q**ATL3EAEV** LKT SQT PQT S TTLFLA **LISV** LGP **PVTGES** gp5

(e) YWAYLPKKPPI LHPVGWGSTD PIRVLTNQTM YLGGS **PDFHG** FRNMSGNVH F

### *Putative post-translational modification motifs*

- N- PKC
- Amidation CK2

**Figure 1.** A. Localization of p14 within MMTV. The env gene is translated into the p73 env precursor.

B. Sequence and putative modification sites of p14. The boxed area is the putative NLS. The shaded sequence includes the epitope recognized by monoclonal antibody M-66

A 2 GSITRDSSDLISLGKDRRPRIALRRKKRREMRKIKTKKKVRRMTTLDPIKEKTA 51  
 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |  
 p 7 GSPTGSSSDLILSGKKQRPHLALRRREMKKKINRKVRRMNILDLIKEKTA 56  
 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |  
 52 WQQLQALISEAEEVLKTSQTPQTSILTLFLAILLSVLGPLPVTC 93  
 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |  
 57 WQHLQALISEAEEVLKTSQTPQTSILTLFLAILLSVLGPLPPVTC 98

Figure 2. Comparison of the amino acid sequence of p14 with the sequence of the human proviral structure reported by Liu *et. al.* (12). The sequence of the proviral structure was translated and the open reading frame corresponding to the N-terminal portion of the ENV precursor protein was compared to the sequence of p14 using the BestFit program of the GCG package. The sequences are 85% identical and 88% similar. The nucleic acid sequences are 95% identical.

Figure 3. Nucleolar localization of p14  
in mouse mammary carcinoma cells  
(Mm5MT) in culture as shown by  
confocal microscopy. Cells were grown  
on polylysine coated slides, fixed and  
permeabilized. p14 was visualized  
using PAb14 (see text) and fluorescent  
anti-rabbit second antibody.

